Nuclear DNA Content Variation and Species Relationships in the Genus Lupinus (Fabaceae)

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The 2C nuclear DNA content has been estimated by flow cytometry in 18 species and botanical forms of the genus Lupinus (family Fabaceae), using propidium iodide as a fluorescent dye. They represented distinct infrageneric taxonomic groups and differed in somatic chromosome numbers. Estimated 2C DNA values ranged from 0.97 pg in L. princei to 2.44 pg in L. luteus, which gives a more than 2.5-fold variation. Statistical analysis of the data obtained resulted in a grouping that supports the generally accepted taxonomic classification of the Old World lupins. The rough-seeded L. princei turned out to be an interesting exception, getting closer to smooth-seeded species. Results of DNA content analyses are discussed with regards to the phylogenetic relationships among the Old World lupins and some aspects of the evolution of the genus.

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Key words: Lupinus, lupin, flow cytometry, propidium iodide (PI), nuclear DNA content, genome size, chromosome number, taxonomy, phylogeny, evolution.

INTRODUCTION

The genus Lupinus Tourn. (family Fabaceae) comprises species of the Old and New Worlds, covering a wide climatic range. There are both wild forms and crops within the genus. Lupins of the Old World are represented by 12 annual species and consist of two distinct groups: (1) Scabrispermae—rough-seeded, closely related wild species; and (2) Malacospermae—smooth-seeded lupins, a heterogeneous group, including also among the wild species three lupin cultivars (Plitmann and Heyn, 1984; Gladstones, 1998).

In the New World there are about 500 taxa of lupins (Dunn, 1984). The species are taxonomically poorly defined; their cytological studies were restricted mainly to chromosome counting. The predominant chromosome number is 2n = 48 and the basic chromosome number x = 6 (Dunn, 1984).

The Old World lupins show a large variability of chromosome numbers. They have a series of different basic chromosome numbers x = 5, 6 (or 9), 7, 8 and 13, as well as different ploidy levels (Pazy et al., 1977). Somatic chromosome numbers of the rough-seeded species range from the lowest numbers for L. cosentinii (2n = 32) and L. digitatus (2n = 36), to L. atlanticus and L. princei with 2n = 38, and L. pilosus and L. palaeantis both having 2n = 42. Gladstones (1998) suggested that the recent increase in the chromosome number in this group was due to individual chromosome duplications or subdivisions as a result of evolution in changeable environments. The smooth-seeded Old World lupin species have higher chromosome numbers ranging from 2n = 40 in L. angustifolius to 2n = 50 in L. albus and 2n = 52 in L. micranthus, L. luteus and L. hispanicus. The type of genome rearrangements, leading to an increase in the chromosome number in this group, is unknown, but a polyploidy of whole ancestral genomes cannot be precluded especially in the L. luteus/L. hispanicus complex, which appears still to be evolving in the cool climate of Europe.

Because of the abundance of botanical forms and their specific eco-geographical distribution, as well as a difficult-to-explain variation in the chromosome number, the phylogeny of lupins is unclear and relationships among contemporary forms are not well determined. Lupins are considered to be of polyploid origin (Wolko and Weeden, 1989; Gupta et al., 1996; Atkins et al., 1998). Polyploidy probably played a crucial role in their evolution, early in the phylogenetic development of their ancestral forms. The ancient character of polyploidy is manifested by the well-established diploid genetic behaviour of lupins (‘chromosomal diploidization’), especially of the Old World species (Wolko and Weeden, 1989; Gupta et al., 1996). During evolution the events of allo- and autopolyploidization, together with other chromosomal changes, probably occurred. As is postulated by Wendel (2000) for most angiosperms, events of duplication followed by genome divergence and the ‘diploidization’ process (possibly at several cycles at various times) might have occurred, resulting in the diversity in chromosome numbers and genome size.

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Recent evidence from DNA analysis (Käss and Wink, 1997; Aïnouche and Bayer, 1998), combined with the earlier lupin evolution hypotheses (Plitmann, 1981; Dunn, 1984; Cristofolini, 1989), indicates the current phylogeny of the genus Lupinus. It assumes a progressive development and branching off – starting with the simple-leaved species of the South American Atlantic centre. They are the most primitive, both in their morphology and in their subtropical warm-temperate adaptation. Secondly, the rough-seeded species evolved. They appeared to be relict descendants of an early northward evolution out of the subtropics, with a specialized adaptation to semi-desert and warm Mediterranean conditions. The Old World smooth-seeded lupins are a further step away from the tropics and deserts to the cooler and wetter northern Mediterranean climate. Finally, the North and South American west coastal species appear to have evolved from the Old World smooth-seeded lupins, or from the same ancestral stock. It suggests a westward long-distance transport across the Atlantic, perhaps a number of times, followed by a local evolution and secondary long-distance migration and spread to South America (Gladstones, 1998).

The nuclear DNA C-value in plants is probably a character under strict genotypic control within defined limits (Bennett et al., 2000a). Nucleotypic correlations with many phenotypic and phenological characters at the level of cell, tissue and organism have been shown. There is an ongoing discussion on factors determining and controlling genome size, and especially on mechanisms responsible for the DNA gain or loss during evolution (Bennett et al., 2000a; Knight, 2002). It seems that the C-value tends to be characteristic for a taxon and constant within many species. Reports on its infraspecific variation have been verified by subsequent studies and the idea of a relative genome size constancy within a species has been recently supported by new evidence (Baranyi and Greilhuber, 1996, 1999; Greilhuber and Obermayer, 1997; Greilhuber, 1998; Bennett et al., 2000b). The usefulness of the C-value, together with other genome traits, in phylogenetic studies at the species, genus and family levels is still being increased (e.g. Martel et al., 1997; Ohri, 1998; Cerbah et al., 1999, 2001; Lysák et al., 1999; Dimitrova and Greilhuber, 2000; Torrell and Vallès, 2001). Nuclear DNA analysis turned out to be very effective in delimiting infrageneric divisions in a number of taxa (Ohri, 1998).

Flow cytometry is a fast and accurate method for the DNA content estimation. Lately it is widely used for establishing the genome size of many plant species (Marie and Brown, 1993; Baranyi and Greilhuber, 1996; Lysák et al., 1999; Bennett et al., 2000b; Joachimiak et al., 2001; Torrell and Vallès, 2001). It has also been successfully applied for the estimation of the nuclear DNA content in some Lupinus species (Obermayer et al., 1999).

In the present study, flow cytometry was used to examine the DNA content values in the Old World lupins, as well as one New World species. This is the first study of the DNA content in the genus Lupinus by flow cytometry, based on data for a large number of species. The results were used to determine the range of variation within and among taxa of the genus and to investigate relationships among the various taxa. Since the majority of the smooth-seeded lupin species has been under cultivation for a long time, the 2C-values of lupin cultivars were compared with their closely related wild botanical forms to determine whether cultivation had any marked effect on the DNA content within the species. Of particular interest was the determination of whether data obtained from the DNA content analysis of species, originating from different centres of diversity, would provide any information on the origin and evolutionary relationships within the genus.

MATERIALS AND METHODS

Plant material

Seventeen species/botanical forms representing all the Old World lupins and one New World species were used in the experiment. Seeds were obtained from lupin gene banks in Poland and abroad (Table 1). Besides the wild lupin species, some lupin cultivars (L. angustifolius, L. albus, L. luteus and L. mutabilis) and their related wild botanical forms (L. cryptanthus and L. linifolius — wild forms of L. angustifolius; L. graecus, L. vavilovii and L. termis—wild forms of L. albus) were analysed. The Old World species represented two taxonomic groups: Malacospermae (smooth-seeded lupins) and Scabrispermae (rough-seeded lupins), consequently divided into seven sections based on the morphological, genetic and serological evidence (Gladstones 1974, 1984; Carstairs et al., 1992).

Flow cytometry

Young lupin leaves were collected from 3-week-old seedlings growing in a glasshouse and prepared for the flow cytometric analysis according to Galbraith et al. (1983) with some minor modifications. Petunia hybrida ‘P × Pc6’ (2C = 2.85 pg) was used as the internal standard for flow cytometry (Marie and Brown, 1993). Lupinus angustifolius (2C = 1.89 pg; this study) served as an intermediate standard for DNA estimation in L. luteus. Plant tissue (of the species in question and of the internal standard) was chopped with a sharp razor blade in a plastic Petri dish with 1 ml nucleic-acid isolation buffer (0.1 M Tris, 2.5 mM MgCl2.6H2O, 85 mM NaCl, 0.1% Triton X-100; pH 7.0), supplemented with propidium iodide (50 μg mL−1) and ribonuclease A (50 μg mL−1). After chopping, the suspension was passed through a 50 μm mesh nylon filter. For each sample, 5000–10 000 nuclei were analysed using a Partec CCA (Münster, Germany) flow cytometer, equipped with an argon laser. Histograms were analysed using a DPAC v.2.2 computer program. Nuclear DNA content was calculated using the linear relationship between the ratio of the 2C peak positions Lupinus/Petunia or L. luteus/L. angustifolius, on the histogram of fluorescence intensities (Fig. 1).

For each species at least 20 measurements of separate nucleus isolation from different plants were made (except a few species for which ten measurements were performed because the material was not available).
Naganowska et al.—Nuclear DNA Content in Lupinus

### Table 1. Origin of Lupinus seeds used in the experiment

<table>
<thead>
<tr>
<th>Section</th>
<th>Species/botanical form</th>
<th>2n</th>
<th>Catalogue accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Old World lupins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scabrispermae</td>
<td>L. pilosus</td>
<td>42</td>
<td>Wt 98656*</td>
</tr>
<tr>
<td>(rough-seeded lupins)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atlanticus</td>
<td>L. luteus</td>
<td>50</td>
<td>Wt 95602*</td>
</tr>
<tr>
<td>Malacospermae</td>
<td>L. graecus</td>
<td>50</td>
<td>Wt 95631*</td>
</tr>
<tr>
<td>(smooth-seeded lupins)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albus</td>
<td>L. mutabilis</td>
<td>48</td>
<td>Wt 1067*</td>
</tr>
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<td>L. pilosus</td>
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</tr>
</tbody>
</table>

| * Gene Bank of Lupinus Genus, Plant Breeding Station, Wiatrowo, Poland (curator: Professor W. K. Święcicki).
| 1 Department of Plant Breeding and Seed Science, Academy of Agriculture, Wrocław, Poland (curator: Professor E. Sawicka-Sienkiewicz).
| 2 Seccção de Forragens, Estação Agronómica Nacional, Oeiras, Portugal (curator: Dr M. da P. Campos-Andrade).
| 3 Centre for Legumes in Mediterranean Agriculture, University of Western Australia, Nedlands, Western Australia 6907, Australia (curator: Dr B. J. Burchell).

### Statistical analysis

A one-factor ANOVA was applied to the DNA content values. This method permits the verification of the hypothesis about a lack of differences between the species (botanical forms). The Gabriel procedure (Gabriel, 1964) was used to test the homogeneity of all groups contained in the set of all species involved in the ANOVA. Significance decisions are based on sums of squares between means within groups, using the same critical value as for the overall F-test. The procedure includes the ANOVA F-test as well as decisions on pair-wise contrasts using Scheffé’s method.

### RESULTS AND DISCUSSION

The results of the 2C DNA content analysis obtained in the present study are presented in Fig. 2. Chromosome numbers of the lupins studied are given in Table 1. The 2C DNA values ranged from 0.97 pg in L. princei to 2.44 pg in L. luteus, which gives an overall variation of more than 2.5-fold. The variation among rough-seeded lupins is 1.7-fold (DNA values from 0.97 to 1.61 pg), whereas among smooth-seeded species it is 2.5-fold (from 0.98 to 2.44 pg). The 2C DNA value for L. mutabilis (the New World species) was 1.90 pg. No significant correlation between DNA content values and somatic chromosome numbers was found for the lupins under study \((r = 0.124)\). The C-value data obtained in this study show good correspondence with those obtained for five species in three previous publications (Bennett and Smith, 1976; Barlow, 1981; Obermayer et al., 1999) as shown in Fig. 2.

ANOVA showed significant differences \((P < 0.01)\) among the species studied, which entitled us to further statistical studies. In order to find interspecific relationships based on DNA content variation, the analysis of homogeneous groups was performed using the results of the variance analysis (Fig. 2). It revealed four groups, corresponding with the present taxonomic classification of the Old World lupins.

1. L. hispanicus subsp. hispanicus, L. hispanicus subsp. bicolor and L. luteus (section Luteus)—closely related species, often defined as a complex.
2. Lupinus angustifolius and two wild botanical forms sometimes considered as its subspecies—L. cryptanthus and L. linifolius, composing the section Angustifolius.
3. Five rough-seeded lupins: L. pilosus, L. palaestinus (section Pilosus) and L. atlanticus, L. cosentinii and L. digitatus (section Atlanticus)—forming a closely related group of species (Gupta et al., 1996).
4. Smooth-seeded wild species: L. micranthus (section Micranthus), L. albus and its three wild botanical forms L. graecus, L. termis and L. vavilovi (section Albus) and L. princei (section Princei).

Lupinus luteus and two subspecies of L. hispanicus, widely treated as a complex, belong to the first group. They have smooth seeds and are often included in the separate section Luteus. Both wild subspecies of L. hispanicus and wild ancestors of L. luteus (a species currently cultivated in
Europe) originate from the Iberian Peninsula. *Lupinus hispanicus* and *L. luteus* have the same chromosome numbers (2n = 52) and are distinguished by the highest 2C DNA values among the lupins studied. Almost the same values of the DNA content (2·1 pg nucleus⁻¹) and the natural inter-fertility common between both *L. hispanicus* subspecies (Gladstones, 1974, 1976) confirm their close genetic relationship (Fig. 2). The stable interspecific hybrid between *L. hispanicus* *ssp. hispanicus* and *L. luteus*, a result of the composed cross combination (Święcicki et al., 1999), also supports a close affinity between *L. hispanicus* and *L. luteus*.

Species of the three remaining groups contain significantly less DNA than those of the first one (Fig. 2). Apart from group A, the Malacospermae lupins form two other groups differing essentially between one another in DNA content. Group B comprises the smooth-seeded species *L. angustifolius* and its wild forms, *L. cryptanthus* and *L. linifolius*. The only representative of the New World, *L. mutabilis*, is close to the smooth-seeded *L. angustifolius* (section Angustifolius), with regard to its DNA content. Group D comprises the smooth-seeded wild lupin species *L. micranthus* and *L. albus* with its wild botanical forms, *L. graecus*, *L. termis* and *L. vavilovi*, but also the rough-seeded species *L. princei*.

The high conformity of 2C-values among the cultivated forms—narrow-leaved lupin *L. angustifolius* ‘Sonet’ and white lupin *L. albus* ‘Wat’—and their wild relatives in the sections Angustifolius and Albus, respectively, suggests a genome similarity between wild ancestors and current cultivars. It seems probable that the long process of domestication and cultivation, especially in the case of *L. albus* (Gladstones, 1998), did not leave any marked effect on the DNA content within each species.

The smooth-seeded wild species *L. micranthus*, the most widespread of the lupins around the Mediterranean Basin, has been identified as a taxon important for the understanding of the relationships between Old World lupins (Wolko and Weeden, 1990a). The biochemical marker analyses indicated its intermediate position among lupin species, which could be a result of hybridization and introgression between smooth- and rough-seeded types or reflect a remainder of the transitional lineage from which the smooth-seeded species were derived. *L. micranthus* and *L. albus* were clustered in a common clade based on ITS (internal transcribed spacer sequence) comparative analyses (Käss and Wink, 1997; Ainouche and Bayer, 1998). The results of this DNA content analysis confirmed a close relationship between these species.
Surprisingly, the DNA content of *L. princei*, the rough-seeded species, was closer to the smooth-seeded species (section Albus), than to the remaining rough-seeded ones (Fig. 2). Interestingly, this African species differs from the rest of the Scabrispermae also in other aspects. According to Carstairs *et al.* (1992), it has the longest chromosomes among the rough-seeded lupins analysed cytologically (preliminary observations do not support a clear difference). Its position within the group is genetically isolated (Gupta *et al.*, 1996). Although morphologically a typical member of the rough-seeded lupins, with the same chromosome number as *L. atlanticus* (2n = 38), *L. princei* has always failed to produce viable seeds when crossed with other species of the group (Carstairs *et al.*, 1992; Gupta *et al.*, 1996). In contrast, the molecular phylogeny analyses of *Lupinus* based on nucleotide sequences of the *rbcL* gene and ITS regions of rDNA (Käss and Wink 1997) showed the Scabrispermae to be an unusually homogenous, closely related evolutionary group, contrary to the wide diversity in their chromosome numbers. Based on DNA content analysis, the rough-seeded species form a clearly distinct, close group among the Old World lupins. However, as mentioned above, *L. princei* seems to be an interesting exception.

Generally, the 2C-values obtained for the Old World lupins confirmed their taxonomic division into Scabrispermae (rough-seeded) and Malacospermae (smooth-seeded). Most of the rough-seeded species form the homogenous group, which is consistent with the results of earlier studies. Their average DNA content puts them between the smooth-seeded Albus and Angustifolius sections. The only exception is *L. princei* which has the lowest 2C-value of all the species analysed. The results obtained also confirmed the heterogeneous character of the smooth-seeded group. Earlier studies showed that the *L. luteus/L. hispanicus* complex is widely separated from the other smooth-seeded species (Nowacki and Jaworski, 1978; Cristofolini, 1989; Wolko and Weeden, 1990b). However, the DNA analysis (Käss and Wink, 1997; Ainouche and Bayer, 1998) suggests a weak grouping of the *L. luteus/L. hispanicus* complex with *L. angustifolius*, and of
L. micranthus with L. albus. This fact and earlier serological (Cristofolini, 1989) and isozyme (Wolko and Weeden, 1990a) evidence suggest that the last two species are genetically closer to the rough-seeded group than the other smooth-seeded species. The 2C DNA values within the Malacospermae group showed a 2.5-fold variation. The chromosome numbers of the smooth-seeded lupins are high and vary among species, but they do not correlate with the DNA content. It is questionable whether such large differences in the DNA content can be a result of individual chromosome duplications or subdivisions. On the other hand, the significant difference in the DNA content found between L. princeps and the rest of the rough-seeded species indicates that this group does not appear to be as homogenous as was previously estimated. This suggests that the variability in the DNA content revealed among the Malacospermae species might be a result of several independent evolutionary lineages from the ancient rough-seeded stock. The similarities in the DNA content between the rough-seeded L. princeps and the smooth-seeded species, L. micranthus and L. albus, could support such a possibility. The inadequate sampling of the American species in the present study makes it impossible to investigate all aspects of recent hypotheses on Lupinus evolution. The similarity of DNA contents between lupins from the Angustifolius section and L. mutabilis (the only New World species included into the analysis) may only suggest their affinity and cannot be considered as proof of a close relationship between these taxa.

This study of the DNA content in Lupinus, the first in this genus with a large number of species, supported the generally accepted infrageneric classification of the Old World lupins and could be useful in a further discussion concerning a possible history of the Lupinus genus evolution.

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LITERATURE CITED


